

OPERATION MANUAL

For



Rotary Culture MW™-(RCMW™)

Continuous Flow Perfusion System

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Table of Contents

- 1.0 General introduction to the Rotary Culture Max™**
- 2.0 Read before using--Limited Warranty**
- 3.0 Getting Started-- Unpacking and Inspection**
- 4.0 RCMW™ preparation required before culture initiation**
- 4.1 System disassembly for washing & sterilization**
- 4.2 Washing the RCMW™ Components after disassembly**
- 4.3 Re-assembly after sterilization**
- 5.0 Troubleshooting**
- 6.0 How to order disposable pre-sterilized parts**
- 7.0 Example Cell Culture Protocols for first time users**

1.0 GENERAL INTRODUCTION TO THE ROTARY CULTURE MAX™

Synthecon, Incorporated is the industry leader in the design and utilization of unique Rotary Cell Culture Systems™ widely applicable in industrial, academic, and aerospace environments. A great variety of cell types from different species have been successfully grown in these systems to date including: osteoblasts, chondrocytes, skeletal muscle, human and murine tumors, primary human hepatocytes, a variety of human explant tissues, and fragile cell cultures unable to be cultivated in any other system (see bibliography at the end of the manual and www.synthecon.com for a continuously updated list). Cells have been successfully grown both in the absence of solid supports or using any of a variety of support structures; i.e., microcarrier beads, collagen macro beads, alginate beads, and various scaffolding materials. Cells subcultured in the unique bioreactor environment have been shown to exhibit unique properties; i.e., enhanced gene expression, enhanced production of bio-products, spontaneous formation of three-dimensional tissue assemblies, etc.

The advanced design **ROTARY CULTURE MAX™ (RCMW™)** represents the next generation of Continuous Perfusion Bioreactors exclusively manufactured by Synthecon, Inc. The RCMW™ rotates the cell culture chamber horizontally to maintain the seeded cells suspended in the culture medium and provides an exceptional cell culture environment that enhances cell growth through the

*absence of air bubbles in the zero head space cell culture vessel,
minimal shear forces,
high mass transfer of nutrients,
effective waste removal, and
efficient oxygenation.*

The RCMW™ incorporates a number of design changes providing increased applicability, durability, and ease of use compared to earlier prototypes. These innovations include:

1. An *advanced design cell culture vessel* incorporating a dialysis membrane-covered perfusion core which provides nutrient and gas exchange with cultured cells without direct exposure to media flow.
2. The new *in-line oxygenator* system provides external gassing of the media insuring a low stress culture environment
3. A new *direct drive rotation system* provides ease of operation and eliminates the older belt driven pulley and gear assembly found in earlier systems.
4. For increased ease of use, access and serviceability, the culture vessel is now rotated in a *cradle roller assembly*. The culture vessel is perfused through rotating fluid couplings located at the vessel endcaps. For removal, simply lift the culture vessel from the cradle for service, thus eliminating the need for manipulation of metal shafts and complex gear assemblies.
5. The media circulation loop now has two 3-way stopcock valves to simplify media management in the circulation loop. The RCMW™ is now equipped with *one media reservoir bottle* located on the rear face of the vertical support.

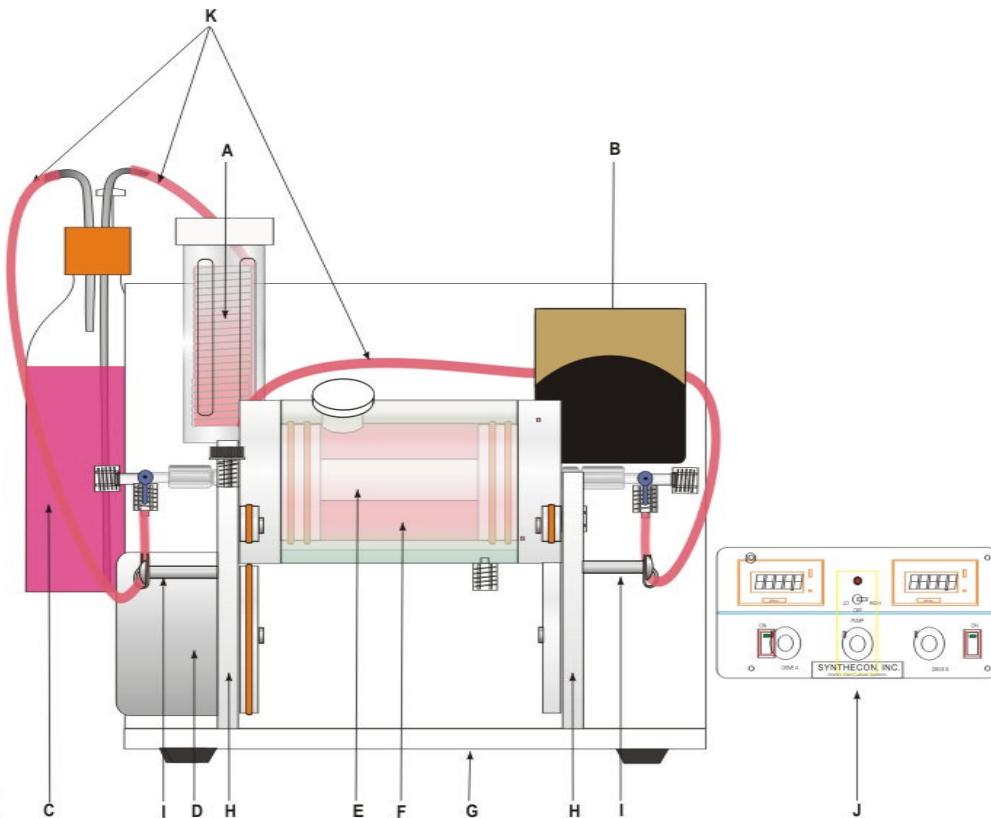


Figure 1

The RCMW™ allows the growth and maintenance of a variety of cell and tissue types in monocultures, co-cultured three-dimensional tissue-like cell aggregates and explants. The RCMW™ accomplishes this by continuously perfusing fresh nutrients and gas, and effectively removing of waste products while maintaining a low shear suspension environment. The large 500 ml media reservoir along with the culture chamber volume, insure an extended culture. The media reservoir being outside the culture chamber, essentially, eliminates the need to remove the growth chamber from the incubator to replenish the medium. The key advantages of a perfused cell culture system include:

1. Ability for long-term un-interrupted cell culture
2. Clear unobstructed viewing of culture chamber environment.
3. Closed loop circulation of fluid, which reduces risk of contamination.

The RCMW™ is composed of the following components shown in [Figure 1](#).

A. Oxygenator assembly- externally facilitates oxygenation/CO₂ exchange for cell and tissue cultures in the vessel. The assembly is mounted on the face of

the vertical plate. The unit contains 46 square inches of surface area within thin wall silicone tubing. The fluid volume is about 10 cc. The oxygenator is easily removed for cleaning/sterilization. DO NOT REMOVE THE TUBING FROM THE HOUSING. It is mounted on the upper left side of the vertical plate of the support assembly.

B. Peristaltic pump- clockwise rotation provides the force to pump culture medium through the culture vessel. It is mounted on the far upper right side of the vertical plate of the support assembly. Lift the tan colored faceplate upward to gain access for insertion of the media circulation tubing. Power is provided to the peristaltic pump via a multi-colored ribbon cable, which is connected at the power supply and then at the pump. To insure proper pumping, the tubing must be aligned in the center of the rotating rollers, and locked into the v-notches on the inlet and outlet sides of the pump. The pump flow rate is adjusted using the panel control knobs on the right front of the power supply. One rpm on the peristaltic pump is approximately equivalent to a flow rate through the circulation tubing of 0.5 ml/minute. Rate of flow is governed by the pore size of the flat filter located on the culture vessel's leeward endcap. Smaller pore sizes (3-10 micron) require reduced flow rates to avoid damage from over-pressurization of the membrane. Higher flow rates can be achieved with larger membrane pore sizes (100 micron). Larger pore sizes must be specified when ordering flat filters.

C. Culture media reservoir- a 500 ml Pyrex glass bottle with orange cap containing a **long stainless steel media supply tube** that extends to the bottom of the bottle, a **short steel media return tube**, and a Whatman micro filter to **vent** the bottle. It is located in the middle of the rear face of the vertical plate of the support assembly.

D. Direct motor drive- used to rotate the culture vessel horizontally at speeds from 2-42 rpm. It is equipped with one extended service life stepper motor (no brushes or gear head). The rotational speed switch, located on the left face of the power supply, has three settings: "Low"- adjustable range of 6-27 rpm; "Off" or "High" -adjustable range of 10-52 rpm.

E. Perfusion Core- the plastic cylindrical core fits in the center of the culture vessel. It is to be covered with a dialysis membrane. Media flows through openings at either end of the core underneath the membrane. Media exchange with the cell compartment occurs through the pores of the dialysis membrane.

F. Culture vessel- culture vessels can range in volume from 25 ml to 1L. The standard size vessel is 125 ml. The vessel is composed of two white polyacetyl **endcaps**. Affixed to the endcaps are white delrin luer fittings for attaching rotating fluid couplings. Assembled, the vessel is comprised of two white endcaps, a perfusion core, and a **clear vessel wall**. The vessel wall

contains two sampling/injection Luer lock ports with rubber septums and drain/fill port with a Delrin plastic plug insert.

- G. **Rotator Base/Assembly Support**- serves to support and rotate the culture vessel. It is constructed of two rectangular white Delrin/Acetyl plastic plates (one horizontal and one vertical) creating an envelope 12" wide X 10" long X 10.5" high. Components are mounted on each of the plates. Located on the vertical plate are the oxygenator assembly, peristaltic pump, and media bottle. The roller cradle assembly is attached to the horizontal base plate. It consists of two white Delrin uprights creating a roller cradle in which the vessel is aligned, supported and rotated.
- H. **Upright roller cradle supports**- vertical white Delrin plastic plates equipped with white Delrin direct-drive disk and roller support disk to maintain the culture vessel in proper alignment in the roller cradle assembly.
- I. **Silicone tubing standoff clips**- be sure tygon tubing is inserted through the clip and does not put strain on the rotating coupler. The rotating coupler must remain parallel with the central axis throughout system rotation
- J. **Power supply**- the blue control box that houses the electronic motor speed controls. The front panel knobs are used to adjust vessel rotation speed, as well as, to control the peristaltic pump. A digital tachometer displays rotation speed and rate of pump flow. A flat, multicolored, ribbon cable connects the rotator base to the power supply control box. The rotational speed of the vessel may be set on "Hi", "Low", or "Off". **THE CONTROL BOX SHOULD NEVER BE PLACED INSIDE AN INCUBATOR.**
- K. **Connecting tubing**- reusable, autoclavable Dow-Corning silicone rubber tubing used to complete the perfusion loop (3/32" inner diameter)

External perfusion loop- consists of media supply/waste bottle, oxygenator assembly, peristaltic pump, media and rotating fluid couplings with three-way valves, connected with reusable, autoclavable, silicon rubber tubing.

2.0 READ BEFORE USING-LIMITED WARRANTY

- Please read, complete and return the Limited Warranty Sheet found on the last page *immediately upon receipt* of your new RCMW™. The Warranty will **NOT BE VALID** unless it is signed and returned to Synthecon, Inc.
- The RCMW™ is currently intended for **RESEARCH USE ONLY** (see warranty).
- Caution: performing any of the following can invalidate the warranty.***
 - Soaking any part of the vessel in bleach, acidic, or basic cleaning solutions can result in absorption of toxic chemicals into the rubber fittings and inhibit cell growth.
 - Abrasive cleaners or strong organic cleaning compounds such as acetone *will destroy* the plastic.
 - Corrosive chemicals such as chromates will damage the metal parts.
 - Do not*** autoclave the rotator base/upright assembly (see Figure 1). Table 1 lists those components that can be autoclaved.
 - Sterilizing the autoclavable components (Table 1) for more than the recommended temperature and time (120°C for 20 minutes).
 - Storage of the rotator base/upright assembly in an incubator **while NOT in use** will corrode the motors eventually resulting in loss of function. ***Synthecon reserves the right to make discretionary determination of the cause of damage to returned rotators and deem whether the repair is covered under the limited warranty.***
 - Placing the power supply/control box inside an incubator will result in loss of function.

Table 1 Methods for sterilizing RCMW™ components

Autoclavable components
120°C, 20 min
Culture vessel
(Delrin core, Delrin drain/fill port, O-rings, two end caps, clear vessel wall)
Silicone rubber tubing
0.2 micron vent filters
medium bottle & cap
oxygenator
male and female type tubing connectors

Do not autoclave
Wipe/rinse with ETOH only
White Delrin cradle support assembly
peristaltic pump
power supply outer cover
Ribbon cable

Disposable Components <i>(discard after each use)</i>
Plastic three way stopcock with luer fittings
Rotating fluid couplings
Syringes
Rubber Septums

3.0 Getting Started- Unpacking and Inspection

The RCMW™ is carefully packaged for shipment to ensure the arrival of an intact, functional unit. Unfortunately, on rare occasions, some damage may incur during handling by the freight carrier.

- Upon receipt, visually inspect each system component closely after unpacking for visible or concealed damage.
- IF DAMAGE IS EVIDENT OR SUSPECTED, DO NOT ASSEMBLE OR OPERATE THE UNIT. Please call Synthecon, Inc. at (800) 853-0740 if you are in the USA. For assistance outside the USA, please call your closest distributor listed at the end of the manual.

4.0 RCMW™ preparation required before culture initiation

Before initiating the growth of cultures, the RCMW™ must be properly prepared.

Disassembly and discard of disposable components
Cleansing/washing of autoclavable components
Rinsing/soaking
Autoclaving appropriate components
Unit re-assembly

CAUTION: ALL RCMW™ COMPONENTS ARE NOT STERILE UPON ARRIVAL EXCEPT THOSE IN STERIL PACKAGES INCLUDING:

Three way stopcock/rotating fluid couplings
Cannulas and Septums
One way valves

The RCMW™ is shipped completely assembled with these non-sterile disposable components attached for instructional use ONLY and must be cleaned and sterilized, or discarded and replaced with pre-sterilized components before use.

4.1 System disassembly sequence for washing & sterilization

Please refer to [Figure 1](#) for proper identification of parts to facilitate disassembly. Remove the following items to wash and autoclave or for replacement with pre-sterilized components *before initializing* a sterile cell culture in the RCMW

Upon receipt of the RCMW™ unit and prior to actual operation-

1. Disconnect disposable rotating coupling from each end of the culture vessel and discard.
2. Carefully remove the culture vessel. Remove each of the white Delrin end caps while holding the vessel (a plastic screw is included to assist with removing the end caps. You will see screw holes in the Delrin end cap. Put the screw in one of the holes and screw in until the end separated from the vessel wall. Remove screw and reinsert into another hole to further separate the vessel from the end cap. A slight unscrewing twist of the end cap will make it easier to remove). Unscrew the white Delrin drain/fill port. Place all parts in a secure area where they cannot be knocked onto the floor and become damaged.
3. Remove tubing from the support clips located on the outer face of the upright support plates of the roller cradle.
4. Disconnect and remove the silicon rubber tubing forming the perfusion loop. Note how the tubing is connected to facilitate re-connection after washing and sterilization. Disconnect the tubing from the peristaltic pump. **NOTE: leave the tubing connected to the orange cap of the media bottle to facilitate re-assembly.**
5. Remove the oxygenator assembly by gently removing the bolt on the bottom of the white bracket. Support the assembly by *using your fingers* while removing the oxygenator to prevent it from falling. **DO NOT REMOVE TUBING FROM OXYGENATOR HOUSING!**
6. Remove the media bottle from the holder in the rear of the vertical plate of the support assembly.

4.2 Washing the RCMW™ Components after disassembly

1. Use a mild liquid soap—a laboratory soap such as Liquinox or equivalent.
2. Soak all components (disassembled culture vessel, oxygenator, media bottle, connecting tubing) in the soap dissolved in warm water for about 10 minutes.
3. The RCMW^a may be washed with a soft brush. Avoid sharp instruments as any damage to the vessel wall can cause progressive cracking after multiple autoclavings.

The oxygenator requires an injection of soapy water using a 20-60 cc size syringe and then allowed to soak.

4. After cleansing, thoroughly rinse the components by first carefully transferring them to a scrupulously clean bucket containing fresh tap or deionized water. (*Do not use a bucket or container that has been used with toxic chemicals or detergents*). Rinse each component under gently running water until all traces of soap are completely removed.
5. The oxygenator will need to be rinsed by injecting water through the tubing or syringe port using a 20-60 cc size syringe several times. REMEMBER: DO NOT REMOVE THE TUBING FROM THE OXYGENATOR HOUSING.
6. **Either of two rinsing protocols can be used.**

Rinsing the components is a critical step since residual soap can be harmful to cell cultures. First, hand rinse all components in high purity, cell culture grade water (i.e., Millipore Milli Q water or the equivalent). Next, either

1. Completely immerse and soak all components in high purity water overnight or
2. Alternatively, rinse components in continuously running high purity (Milli Q or equivalent not deionized)water for 45-60 minutes.

(Do not forget to use a syringe to rinse the oxygenator) least 6 times with high purity water).

7. The perfusion core with the membrane attached must be autoclaved separately from the other components. A piece of dialysis membrane is cut to a length slightly larger than the distance between the two inner grooves of the perfusion core. The membrane is hydrated in deionized water for approximately 10 minutes. The membrane is opened by gently rubbing between the thumb and forefinger. After opening, the membrane is gently pulled over the perfusion core (This process can be assisted with a **blunt** forceps being careful to pull to membrane at the end. If the membrane is punctured, it must be replaced). The dialysis membrane is held in place with o-rings, which are installed with the conical plastic o-ring tool. The o-ring is slipped over the narrow end of the tool and stretched by pushing toward the large end. The tool is then placed over the end of the perfusion core and the o-ring rolled on to the core and into the groove to secure the membrane. After placing o-rings at both ends, any excess membrane can be trimmed. After the membrane is mounted on the core it can be sterilized by one of several methods depending on the composition of the membrane. Regenerated cellulose membranes may be autoclaved at 121° C for 15 min. if completely immersed in deionized water. Cellulose ester membranes may be sterilized in 70% ETOH, preferably overnight. After exposure to ETOH,

the membranes should be rinsed with sterile water or media. PVDF membranes can be autoclaved dry at 121° C.

The Perfusion core with the mesh membrane can be autoclaved with other components as stated in 9

8. Prepare components for autoclaving by wrapping (i.e., autoclave bags or aluminum foil) and secure with autoclave tape. The culture vessel should be autoclaved disassembled.

REFER TO THE LIST IN TABLE 1 FOR THE COMPONENTS THAT CAN BE AUTOCLAVED.

9. Autoclave components at 120°C for 20 minutes. A second cycle of autoclaving at 120°C for 20 minutes can be performed if desired but should be unnecessary.
CAUTION: *Higher temperatures and longer autoclave times must be avoided as these can damage components and void the warranty.*
10. **For ETO (Gas) Sterilization,** wash the RCMW™ components as described above. Open all ports and wrap the system in a gas permeable material. After gas sterilization, it is not necessary to perform an “air soak”. In the hood, fill the system and culture vessel with sterilized high purity water that has been made slightly acidic with a few drops of concentrated hydrochloric acid (HCL). Connect the waste line to the culture vessel and circulate the acidic water for at least 8 hours (overnight). ETO is more soluble in acid water and will be completely removed using this method.

Since gas sterilization is performed at lower temperatures, this will likely extend the life of the culture vessel. However, ETO is toxic to cell cultures and residual ETO will be present in the system. It is, therefore, necessary to rinse carefully and completely as described above in step 6.

4.3 Re-assembly after sterilization

Refer to Figure 1 for guidance in re-assembly.

1. Allow the sterilized components to cool to room temperature before attempting re-assembly. Failure to do so will result in misalignment of components due to thermal expansion.

Take all sterilized components to a sterile work area; i.e., a laminar flow biological safety cabinet (sterile tissue culture hood).

Carefully unwrap the culture vessel wall and endcaps. Install one endcap in the wall. Pick up the perfusion core with a sterile forceps and place it in the counterbore in the inner face of the endcap. Take the other endcap and place it onto the perfusion core. Press the endcaps together until they are flush with the clear vessel wall. Try to avoid touching the fill port or sampling ports. If these areas are accidentally touched, wipe them immediately with a sterile alcohol swab. Press the plug into the fill port and affix rubber septums to the luer sampling ports. Attach a disposable, pre-sterilized rotating coupling and three-way valve to both ends of the culture vessel. Place the culture vessel in the roller cradle of support assembly/rotator base (in the hood).

To complete assembly of the culture vessel prior to filling with media, attach the autoclaved oxygenator assembly and tubing as shown in figure 1

2. **Filling the Culture vessel with media.** Fill the media bottle with the desired volume of media. Remove the fill port plug on the vessel wall and place it on a alcohol swab. Fill the cell chamber of the vessel with media and cells until it reaches the fill port. Microcarriers or other scaffolding material can also be added at this time. Replace the fill port plug being careful not to contaminate the plug.
3. Open the cover of the peristaltic pump so that the tubing is not pinched. Place a 20-60 ml sterile syringe on one of the luer ports on the rotating coupling and turn the valve to open that port. Using the syringe, prime the tubing by drawing media from the bottle until it reaches the syringe. Rotate the valve so that the media flow is directed into the vessel through the perfusion core. Close the cover of the peristaltic pump. Place a 5 or 10 ml syringe with a plastic canula on the sampling port (wiping the septum with a sterile alcohol swab whenever it is to be punctured is recommended). Maneuver the bubble in the cell chamber beneath the sampling port and draw the bubble out. Media will be pulled through the membrane to replace the volume of the bubble. During the culture, if bubbles appear in the vessel, they can be removed by this procedure. The tubing directly attached to the rotating coupling should be inserted into the support clips to prevent the rotating coupling from wobbling, which will cause excessive wear and result in failure of the coupling. The system is now ready to be placed in an incubator and connected to the power supply.

4. Connect the ribbon cables from the power supply to the peristaltic pump and the rotator. The cables can be run between the door and the gasket. The rotational speed is usually set to 10-12 rpm initially. If visible aggregates are formed, the speed should be adjusted upward to maintain the aggregates in suspension without touching the wall of the vessel. The pump speed should be determined empirically according to the metabolic requirements of the cells being cultured. Media changes can be done by simply changing the media bottle in the flow loop to one with fresh media.

No special medium formulations are required for the growth of cells in the RCCS. Each cell type and application is unique. Cell culture medium formulations that you have previously used successfully with other cultivation methods (i.e., petri dishes, flasks, roller bottles, etc.) have generally been found to be appropriate for the RCCS. See the Bibliography and SYNTHECON website www.synthecon.com (bibliography periodically updated) for medium formulations successfully used in the past with the RCCS.

Selected list of type and cell numbers successfully cultured in the Rotary Cell Culture System™

Type of cells	cells/ml inoculated	Reference
Chondrocytes	5-6 X 10 ⁵	Baker & Goodwin, 1997
Human intestine mesenchymal	2 X 10 ⁵	Goodwin et al., 1993
Thyroid	4 X 10 ⁵	Martin et al., 2000
Rat PC12	5.5 X 10 ⁵	Lelkes et al., 1998
LNCaP human prostate	2 X 10 ⁵	Zhau et al., 1997
LN1 mixed mullerian human ovarian cancer	2 X 10 ⁵	Goodwin et al., 1992
Human cervical primary tumor	2 X 10 ⁵	Chopra et al., 1997
16 different tumor cell lines	5-20 X 10 ⁵	Ingram et al., 1997
MIP 101 human colon cancer	3 X 10 ⁵	Francis et al., 1997
HepG2 human hepatoblastoma	1 X 10 ⁶	Khaoustov et al., 1999
HT-29 colon adenocarcinoma	2 X 10 ⁵	Goodwin et al., 1992

5.0 Troubleshooting

Problem	Possible cause/solution
Bubbles present in cell culture vessel	Check for loose connection tubing Incubator could be dry causing excessive evaporation Ensure that the media bottle contains medium
Culture medium leaking	Check valves, couplings, and tubing for tightness of fit Replace if defective
Medium fails to recirculate	Check for proper positioning of steel tube below the fluid level in the media bottle Check position of three way valves and rotating coupling Make certain peristaltic pump is plugged in and speed control set on power supply Insure that tubing is correctly installed in the roller assembly of the peristaltic pump
Vessel rotation is inconsistent	Check that vessel is properly situated in the roller cradle and is not bound up or jammed. The vessel can only achieve rotation if it is in contact with the rotating disk drive located on the inside of the left upright cradle support. Check to insure that the RPM are optimal for the selected high/low range of the power supply
Perfusion tubing twisting	Check that rotating coupling was installed and aligned appropriately with the tubing support clips and that it is turning without an orbital rotation. Replace and align correctly if defective
Culture vessel will not fill	Check position of three-way stopcock valves Look for crimp in tubing at inflow endcap
Oxygenator tubing removed from inside of oxygenator cylinder core	Return to Synthecon for repair
Media leaking from filter on top of waste bottle	Empty waste bottle and replace filter unit

6.0 How to order disposable pre-sterilized parts

Contact Synthecon (713-741-2582 or rccs@synthecon.com) or telephone contract distributors for information on ordering disposable pre-sterilized parts.

7.0 Example Cell Culture Protocols for first time users

It is often instructional for first time users of the system to become acquainted with the RCCS by first using the system with an established protocol before proceeding to actual experiments.

If microcarrier beads or scaffolding will be used, they should be prepared first for inoculation into the vessel *according to the manufacturer's instructions*. An example protocol for microcarrier beads is provided below.

Pharmacia Cytodex 3 Microcarrier Beads

The properties of these beads are as follows: a density of 1.04 g/ml, 175 μm size, a swelling factor of 15 ml/g dry weight, and 3×10^6 microcarriers/g dry weight.

Cytodex 3 consists of a layer of denatured collagen coupled to dextran beads. It is the microcarrier of choice for cells known to be difficult to grow in culture, for differentiated cell culture systems and cells with an epithelial-like morphology. It is commonly used as a general purpose microcarrier.

1. Weigh 1 gram of the dry microcarrier beads on a suitable balance and place into a 100 ml clean glass bottle. (Bottle should be pretreated with a siliconizing agent according to the manufacturer's instructions or excessive loss of beads will occur due to adherence to the glass).
2. Add 50-100 ml of 1X Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) to the bottle and incubate at room temperature for at least 3 hours to allow the beads to swell.
3. Remove supernatant by aspirating with a Pasteur pipette and vacuum source.
4. Wash microcarriers once with gentle agitation for a few minutes with PBS w/o Ca^{2+} and Mg^{2+} . Use 30-50 ml/g Cytodex of PBS.
5. Discard PBS and replace with fresh PBS w/o Ca^{2+} and Mg^{2+} adding 30-50 ml/g Cytodex of PBS.
6. Sterilize by autoclaving at 115°C, 15 min, 15 psi. Make certain that the cap of the bottle is loose before sterilization. **CAUTION: If microcarriers are autoclaved at higher temperatures and/or longer time intervals, beads may turn brown and performance affected.**
7. Prior to use, sterilized microcarriers are allowed to settle, the supernatant removed by aspiration as above and microcarriers rinsed in warm 37°C culture medium (serum is not required in culture medium).

8. Microcarriers are now ready to use for culturing and are extremely stable. Cytodex that has been hydrated and sterilized as above can be stored sterile in PBS for at least two years at 4°C.

Practice Protocol for Cell Culture using Cytodex 3 microcarrier beads

Perform procedures in a biological safety cabinet (sterile tissue culture hood).

1. A commonly available cell line to use for practice is baby hamster kidney (BHK-21). These can be obtained from the American Type Culture Collection (Rockville, MD) or colleagues. Alternatively, the same protocol can be used with MCF-7, a breast cancer cell line, also available from ATCC.
2. Before beginning, obtain a sterilized Synthecon cell culture vessel and rinse with culture medium or phosphate buffered saline as described in Section 4.2.6.
3. Remove BHK-21 cells from culture flasks or dishes: Remove culture medium, add 0.25% trypsin/0.075% EDTA, incubate for 3-5 minutes. Check at minimum time to determine if cells are detaching from flask/dish by observation with an inverted microscope. ***Be careful not to over expose cells to trypsin/EDTA as this can result in loss of cell viability.***
4. After determining that the cells are detaching from the culture flask/dish by observation in an inverted microscope, add sterile, pre-warmed DMEM culture medium (containing 10% fetal bovine serum and penicillin/streptomycin or other antibiotic).
5. Remove the cells from the culture flask/dish by vigorously pipetting the medium (with the assistance of a Pipet Aid device) across the entire cell growth surface at least 5 times. This ensures that all cells are removed and facilitates creation of a single cell suspension. Inadequate pipetting of the cell suspension will result in a clumpy cell suspension which will not produce optimum results.
6. Place cell suspension in a sterile tube. Determine cell number using either a Coulter counter or hemocytometer.
7. Inoculate cells at a final concentration of 2-4 X 10⁵/ml and microcarriers at 5 mg/ml (4,000 beads/mg) according to instructions in Section 5.
8. Place inoculated vessel carefully onto the rotator base. Place rotator base into the culture incubator. Make certain that the ribbon cables are connected between the rotator base and power supply and that the power supply is plugged in. Begin the vessel rotation at a speed of 10-12 rpm.

9. The day after culture initiation, take a sample. If available, use a Beckman glucose analyzer and blood gas analyzer to assess glucose use, dO₂, dCO₂, and pH. Part of the sample should be placed in a small petri dish or on hemocytometer for observation. Cells should be visibly attached to the beads and some beads may be aggregated together in groups of two or three.
10. Day 3- Change medium according to procedures in Section 6 above and take another cell sample. Cell viability can be checked using trypan blue dye exclusion assay (Goodwin et al., 1992).
11. Day 4- A mixture of bead aggregates ranging from 2-16 should be seen.

Practice Protocol for Cell Culture without solid support

1. Before beginning, obtain a sterilized Synthecon cell culture vessel rinse with culture medium or phosphate buffered saline as described in Section 4.2.6
2. Remove cells from culture flask/dish as described in the protocol for microcarrier beads above.
3. Determine the number of cells/ml using a Coulter counter or hemocytometer. Inoculate 8-1.0 X 10⁶ cells/ml into the vessel.
4. Place inoculated vessel carefully onto the rotator base. Place rotator base into the culture incubator. Make certain that the ribbon cable is connected between the rotator base and power supply and that the power supply is plugged in. Begin the vessel rotation at a speed of 10-12 rpm.
5. The day after culture initiation, take a sample according to the procedures described in Section 7 above. If available, use a Beckman glucose analyzer and blood gas analyzer to assess glucose use, dO₂, dCO₂, and pH. Part of the sample should be placed in a small petri dish or on hemocytometer for observation. Cells should have aggregated and formed loose aggregates.
6. Change medium on days 2 and 4. Loose aggregates will be observed to condense and may form rounded structures.

Equipment Usage Rights

SYNTHECON™ Inc. grants the purchaser a non-exclusive right to use the Rotary Cell Culture System equipment solely for the purpose of conducting research and specifically excluding use of this equipment for any purpose other than research. Synthecon technology is not intended for use on/in humans. Any desire by end user to manufacture commercial products in Synthecon, Inc. technology will require the end user to obtain a *User's License* from the National Aeronautics and Space Administration and/or Synthecon, Inc. Its use must comply with all laws, ordinances, and regulations relating to the possession, use, or maintenance of the equipment, including registration and/or licensing requirements, if any.

Patents in Force

The Rotary Cell Culture System™ is protected by patents exclusively licensed from the National Aeronautics and Space Administration (NASA) and patents owned by Synthecon Inc., with others pending. The patents Synthecon Incorporated operates under are listed below:

- Patent number 5,437,998 "GAS PERMEABLE BIOREACTOR AND METHOD OF USE" Patent issued August 1, 1995
- Patent number 5,665,594 "GAS PERMEABLE BIOREACTOR AND METHOD OF USE" Patent issued September 9, 1997
- Patent number 5,702,941 "GAS PERMEABLE BIOREACTOR AND METHOD OF USE" Patent issued December 30, 1997
- Patent number 5,763,279 "GAS PERMEABLE BIOREACTOR AND METHOD OF USE" Patent issued June 9, 1998
- Patent number 4,988,623 "ROTATING BIO-REACTOR CELL CULTURE APPARATUS" Patent issued January 29, 1991
- Patent number 5,026,650 "HORIZONTALLY ROTATED CELL CULTURE SYSTEM WITH A COAXIAL TUBULAR OXYGENATOR" Patent issued June 25, 1991
- Patent number 5,153,131 "HIGH ASPECT RATIO VESSEL AND METHOD OF USE" Patent issued October 6, 1992
- Patent number 5,155,035 "METHOD FOR CULTURING MAMMALIAN CELLS IN A PERFUSED BIOREACTOR" Patent issued October 13, 1992
- Patent number 5,153,133 "METHOD FOR CULTURING MAMMALIAN CELLS IN A HORIZONTALLY ROTATED BIOREACTOR" Patent issued October 6, 1992
- Patent number 5,998,202 "MULTIPLE CHAMBER DIFFUSION VESSEL" Patent issued December 7, 1999
- Patent number 5,989,913 "CULTURE VESSEL FOR GROWING OR CULTURING CELLS, CELLULAR AGGREGATES, TISSUES AND ORGANOIDS AND METHODS FOR USING THE SAME" Patent issued November 23, 1999

Alterations

Alteration of the equipment voids the warranty on this equipment. In no case shall SYNTHECON™, Inc. be responsible for any modifications or alterations to this equipment performed by anyone other than SYNTHECON™, Inc.

IMPORTANT NOTICE

Limited Warranty: Limited Liability

SYNTHECONTM Inc. warrants that, for one year, under normal operating conditions and use, this equipment will be free from defects of materials and workmanship. SYNTHECONTM Inc. will repair or replace defective parts at our option. Contact SYNTHECONTM Inc. immediately upon discovery of a defect. SYNTHECONTM will provide you with a return authorization number and shipping instructions.

Components

Oxygenator Membrane

The oxygenator membrane is a very delicate component consisting of silicone rubber, .005 inches thick, covering a polyester cloth backing. Care and attention should be given to the membrane during cleaning, sterilization, and removal of cultured material. Synthecon reserves the right to make discretionary determination as to the cause of damage with returned oxygenators, and deem whether the repair is covered under the Synthecon Limited Warranty. See Operators Manual for appropriate procedures.

Rotator Base

Storage of the Rotator Base in an incubator while not in use will result in damage to the rotator components. Synthecon reserves the right to make a discretionary determination as to the cause of damage with returned rotators, and deem whether the repair is covered under the Synthecon Limited Warranty.

The equipment must be used and operated in a careful and proper manner. In no event shall SYNTHECONTM Inc. or its suppliers be liable for any indirect, special, or consequential damages, including but not limited to, loss of cells, medium, data, labor or equipment incurred by the purchaser or any third party arising from the use of, or inability to use this equipment.

Service

For service during and after the expiration of the warranty, contact SYNTHECONTM, Inc. at (713) 741-2582 during 9 a.m. to 5 p.m., US Central Time Zone. Equipment being returned for service should be shipped to: Synthecon,- Customer Service Dept, 8044 El Rio, Houston, TX. 77054. Please include a short description of the problem, service required or reason for the return. Please pack equipment being returned in sturdy containers with adequate packing materials. Synthecon will not be liable for damage sustained during shipment.

SYNTHECONTM, Inc. also provides biology and engineering contract support services. Special custom designed equipment can be built to meet the customer's needs. Customers can provide cell samples of their cell and tissue lines, and SYNTHECONTM Inc. will conduct growth and feasibility studies of the customer's cells on a contract fee basis. Sub-licenses are available which would include design, scale-up, and manufacture of production equipment.

Copying and Sale

Duplication, modification or sale of copies of this equipment is prohibited. This equipment is patented by the U.S. Government. SYNTHECON™ Inc. holds the exclusive licenses to these patents.

Acceptance of Equipment

The purchaser shall inspect the equipment delivered and immediately notify the seller of any discrepancies with the equipment. If the purchaser fails to provide notice in writing within 14 days after the delivery of the equipment, the purchaser will be presumed to have accepted the equipment. The acceptance and use of this equipment constitutes an agreement upon the purchaser's part to the usable condition of the equipment.

Refurbished Products

Refurbished products carry a separate warranty; this warranty does not apply. For details of the refurbished product warranty, please refer to the refurbished product warranty information packaged with each refurbished product.

WARRANTY WILL NOT BE VALID IF IT IS NOT SIGNED AND RETURNED.

Warranty valid to original purchasers only. Please sign and return by mail immediately to: **For international locations see the last page of this manual**

Synthecon, Inc.
Customer Service Department
8044 El Rio
Houston, TX 77054

Purchaser: _____

Institution/Organization: _____

Purchase Date: _____

Invoice/PO#: _____

Model #: _____

Serial Number(s) _____

PLEASE NOTE THAT THIS AND THE NEXT PAGE ARE FOR YOUR RECORDS. PLEASE USE THE YELLOW PAGE THAT ACCOMPANIES THIS MANUAL FOR WARRANTY REGISTRATION!

IMPORTANT NOTICE

PLEASE READ AND COMPLETE THE SYNTHECON LIMITED
WARRANTY ON THE LAST PAGE

RETURN THE YELLOW COPY IMMEDIATELY

WARRANTY WILL NOT BE VALID IF NOT COMPLETED AND RETURNED

WARRANTY VALID TO ORIGINAL PURCHASERS ONLY

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WARRANTY VALID TO ORIGINAL PURCHASERS ONLY

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